BioSystems 113 (2013) 66–71

BioSystems

journal homepage: www.elsevier.com/locate/biosystems

Research article

Construction of bacteria–eukaryote synthetic mutualism

Isao Kubo[a], Kazufumi Hosoda[a], Shingo Suzuki[a], Kayo Yamamoto[a], Kumiko Kihara[b], Kotaro Mori[b], Tetsuya Yomo[a,b,c,*]

[a] Department of Bioinformatic Engineering, Graduate School of Information Science and Technology, Osaka University, 1-5 Yamadaoka, Suita, Osaka 565-0871, Japan
[b] Department of Frontier Biosciences, Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan
[c] Exploratory Research for Advanced Technology (ERATO), Japan Science and Technology Agency, 1-5 Yamadaoka, Suita, Osaka 565-0871, Japan

ARTICLE INFO

Article history:
Received 13 April 2013
Received in revised form 14 May 2013
Accepted 16 May 2013

Keywords:
Mutualism
Symbiosis
Syntrophism
Synthetic ecology
Establishment
Bacteria–eukaryote mutualism

ABSTRACT

Mutualism is ubiquitous in nature but is known to be intrinsically vulnerable with regard to both population dynamics and evolution. Synthetic ecology has indicated that it is feasible for organisms to establish novel mutualism merely through encountering each other by showing that it is feasible to construct synthetic mutualism between organisms. However, bacteria–eukaryote mutualism, which is ecologically important, has not yet been constructed. In this study, we synthetically constructed mutualism between a bacterium and a eukaryote by using two model organisms. We mixed a bacterium, *Escherichia coli* (a genetically engineered glutamine auxotroph), and an amoeba, *Dictyostelium discoideum*, in 14 sets of conditions in which each species could not grow in monoculture but potentially could grow in coculture. Under a single condition in which the bacterium and amoeba mutually compensated for the lack of required nutrients (lipoic acid and glutamine, respectively), both species grew continuously through several subcultures, essentially establishing mutualism. Our results shed light on the establishment of bacteria–eukaryote mutualism and indicate that a bacterium and eukaryote pair in nature also has a non-negligible possibility of establishing novel mutualism if the organisms are potentially mutualistic.

1. Introduction

Organisms rarely live alone in nature, and interactions between different species are common (Begon et al., 1996). These interactions are often mutually beneficial, a state known as mutualism, and such relationships are ecologically important (Boucher, 1985; Boucher et al., 1982; Herre et al., 1999). In particular, the establishment and evolution of mutualism are important issues in ecology because mutualism is well known to be vulnerable with regard to both population dynamics and evolution (Ferriere et al., 2002; Herre et al., 1999; Sachs and Simms, 2006). For example, the organisms involved in obligate mutualism can become extinct from lack of interaction because of a decrease in population density, and mutualism can shift to parasitism through the emergence of a cheater during evolution. The histories of the establishment and evolution of natural mutualism have been estimated by analyzing extant organisms using molecular biology and bioinformatics techniques, including phylogenetic analyses (Althoff et al., 2012; Backhed et al., 2005; Bascompte et al., 2006; Cook and Rasplus, 2003; Merckx and Bidartondo, 2008; Ramirez et al., 2011; Schardl and Craven, 2003). However, it is difficult to understand the processes that drove the extinction of organisms (Sachs and Simms, 2006) such as those that encountered a potentially mutualistic partner but failed to establish mutualism or those that once established mutualism but subsequently became extinct through the emergence of cheaters. Thus, our knowledge of mutualism is inevitably biased toward successful examples. To truly understand the difficulty that organisms experience in establishing and maintaining mutualism, the experimental reconstruction of these processes is a useful strategy.

Experimental studies using synthetic ecosystems have suggested that it is feasible for organisms to establish novel mutualism. By constructing synthetic ecosystems that are analogous to mutualism through the use of two species or strains that do not naturally interact, it is possible to simulate their encounter, establishment and maintenance of their mutualism, and the breakdown of their mutualism (Mee and Wang, 2012; Momeni et al., 2011; Tanouchi et al., 2012; Wintermute and Silver, 2010a). Several studies using synthetic mutualism have indicated the difficulties in constructing mutualism in defined cases. This information may be useful

* Corresponding author at: 1-5 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 4171; fax: +81 6 6879 7433.
E-mail address: yomo@ist.osaka-u.ac.jp (T. Yomo).

0303-2647
http://dx.doi.org/10.1016/j.biosystems.2013.05.006

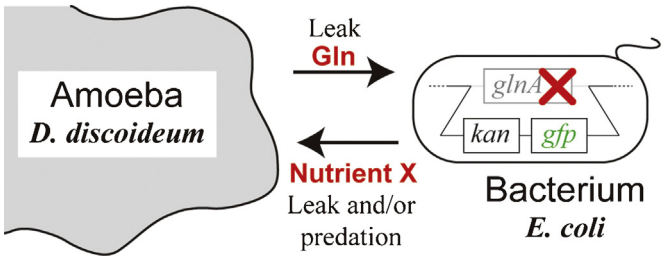

Fig. 1. A scheme of our SOBEM. Both the amoeba and the bacterium can grow in coculture only when they supply a sufficient amount of Gln and nutrient X, respectively (or substitutes that complement the lack of those nutrients).

in determining how difficult it is for organisms to establish mutualism. Shou et al. (2007) pioneered the construction of synthetic mutualism; this interaction was constructed between two genetically engineered auxotrophic yeasts, each lacking a gene essential for the biosynthesis of a nutrient. The study showed that a simple genetic modification resulting in the overexpression of a gene leads to the establishment of synthetic mutualism. In another mutualistic interaction between two different bacterial species studied by Harcombe (2010), the enhancement of the nutrient supply capacity of both bacteria was also necessary for the establishment of mutualism. By mixing 2 of 46 auxotrophic strains of *Escherichia coli*, Wintermute and Silver (2010b) showed that 17% of the 1035 tested pairs increased in population because of the interaction. Using one of those pairs of nutrient auxotrophs, we have shown that the

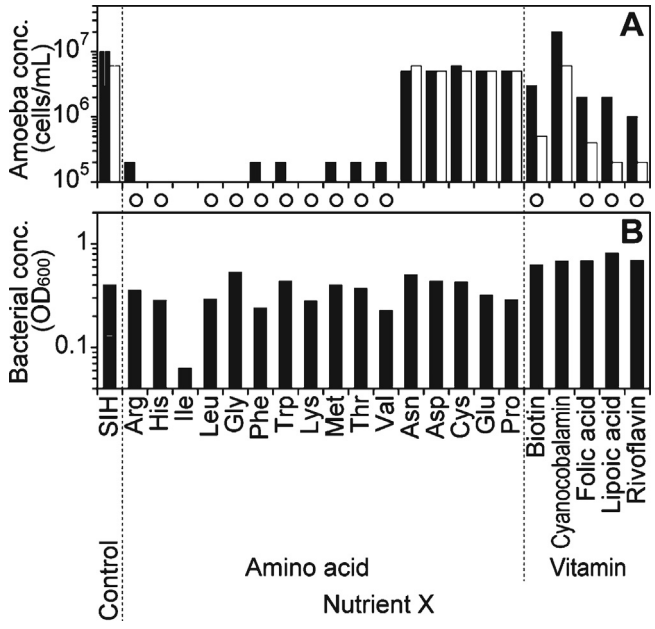

Fig. 2. The growth of the amoeba and the bacterium in monoculture in the absence of each nutrient X. (A) The results of the monoculture of the amoeba in SIH-X. For X to satisfy the requirement, the amoeba must not grow in SIH-X. The omitted nutrient X in each culture is indicated at the bottom of the figure. The amoeba, pre-cultured in SIH, was inoculated in SIH-X at an initial cell concentration of 2×10^5/mL, and the cell concentration was determined after one week (black bar). The amoeba was then transferred to the same medium at the same initial cell concentration, and the cell concentration was determined after 6 or 7 days (white bar). (B) The results of the monoculture of the bacterium in SIH-X + Gln. For X to satisfy the requirement, the bacterium must grow in SIH-X + Gln. The bacterium, pre-cultured in SIH + Gln, was inoculated in SIH-X + Gln at an initial optical density of 0.02 at 600 nm (OD_{600}), and the OD_{600} was determined after 1 day. The symbols ○ between A and B denote the selected candidates. These results are reasonably consistent with existing knowledge (see text), although the experiments have not been replicated.

E. coli populations grew continuously, rapidly changing to more cooperative phenotype (Hosoda et al., 2011; Hosoda and Yomo, 2011). Although the generality has not been sufficiently confirmed, these studies suggest that (i) certain enhancements of cooperative behavior allow the construction of novel mutualism and (ii) novel mutualism can be constructed merely by mixing two populations if approximately one dozen pairs are tested. These results suggest that organisms can with a non-negligible possibility establish mutualism in nature merely through encountering each other. However, mutualism between bacteria and eukaryotes, which is ecologically important, has not yet been constructed.

In nature, bacteria–eukaryote mutualism is commonly observed (Begon et al., 1996; Douglas, 1994; Tarkka et al., 2009), and several studies have reported experimental ecosystems containing bacteria and eukaryotes (Hekstra and Leibler, 2012; Kihara et al., 2011; Matsuyama et al., 2004; Nakajima et al., 2009; Sano et al., 2009; Todoriki et al., 2002a; Tsuchiya et al., 1972; Yamada et al., 2008). However, it remains unclear whether these relationships constituted mutualism, because it is difficult to experimentally determine whether interactions are beneficial if the relationship is not obligate. In contrast, if the relationship is obligate for both species, the interaction must be beneficial to both species. Once synthetic mutualism has been constructed between a bacterium and cells of a mammal (Weber et al., 2007). However, the mammalian cell is no longer an individual mammal, and this situation may have reduced ecological relevance. Accordingly, the construction of synthetic obligate bacteria–eukaryote mutualism (SOBEM) is valuable for investigating the possibility of establishing novel bacteria–eukaryote mutualism in nature.

In this study, we constructed a SOBEM by mixing two model organisms and testing 14 interaction patterns. More precisely, we mixed a bacterium, *E. coli*, and an amoeba, *Dictyostelium discoideum*, under 14 conditions in which both species could not grow in monoculture but potentially could grow in coculture. When *E. coli* and *D. discoideum* mutually compensated for the lack of required nutrients, lipoic acid and glutamine (Gln), respectively, both species grew continuously through several subcultures, essentially establishing mutualism. Our results shed light on the establishment of bacteria–eukaryote mutualism and indicate that a bacterium and eukaryote pair in nature has a non-negligible possibility of establishing mutualism through their encounter if the pair is potentially mutualistic.

2. Materials and methods

2.1. Strains and culture conditions

For the bacterium, we used a green-labeled glutamine auxotrophic *E. coli* strain OSU6 (ΔglnA::(Kan^r)P_{tetA}-*gfpuv5* F$^-$ *endA1 gyrA96 thi-1 hsdR17*(r_K^-, m_K^+) *supE44 relA1*) (Yamada et al., 2008), which is a derivative of *E. coli* DH1 (obtained from the National BioResource Project, National Institute of Genetics, Shizuoka, Japan). For the amoeba, we used an aggregation-defective cellular slime mold *D. discoideum* strain HS175 (erkB−) (Segall et al., 1995), which was a kind gift from Dr. Mineko Maeda of Osaka University. This mutant lacks lipopolysaccharide-dependent enhanced bactericidal activity, which has been shown to be dependent on the MAPK ErkB (Walk et al., 2011), although the predation of the bacterium by this mutant was actually detected (Fig. S1). All of the cultures were grown at 22 °C in a synthetic medium, SIH (Han et al., 2004), or its derivatives, as described in the corresponding text for each experiment. The Gln concentration was 2 mM in all of the Gln-containing media. The antibiotic kanamycin was added at 25 μg/mL to each culture; ampicillin was also added at 50 μg/mL for the amoeba monocultures. The cultures were static (for the amino acids as nutrient X in Figs. 2 and 3) or agitated (for the vitamins as nutrient X in Figs. 2 and 3; 180 rpm rotation in a 300 mL conical flask with 50 mL culture liquid). Before culturing, we washed the bacterium and the amoeba using centrifugation (twice at 4820 × *g* for 5 min and at 890 × *g* for 5 min, respectively) with phosphate buffer (8.9 mM KH_2PO_4 and 2.5 mM NaH_2PO_4; pH 6.5) to exclude the carry-over of supplements from the preculture.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.biosystems.2013.05.006.

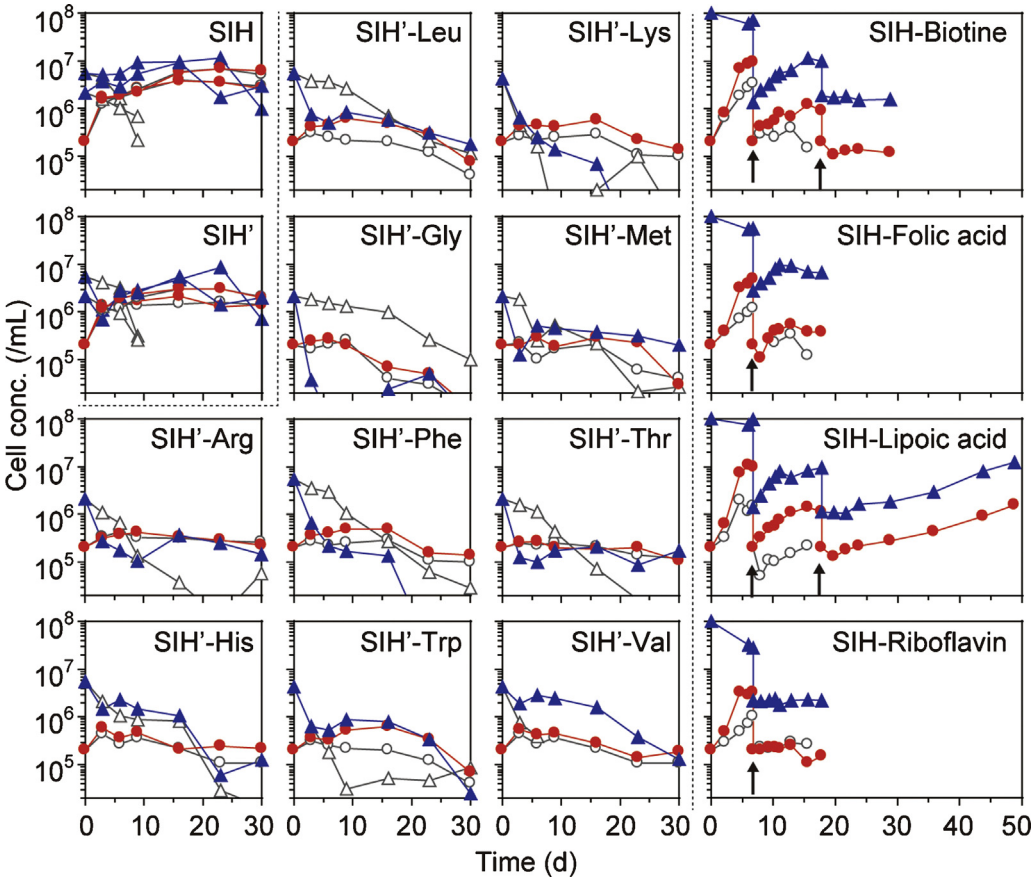

Fig. 3. The population dynamics of the cocultures and monocultures of the amoeba and bacterium in the absence of nutrient X. The cell concentrations of the amoeba and bacterium in the cocultures are depicted as red-filled circles and blue-filled triangles, respectively. The population dynamics in the monoculture of each species are depicted as gray open circles and triangles for the amoeba and the bacterium, respectively. The organisms cocultured in the absence of the vitamins were transferred at the time denoted by the black arrows. We transferred all the cocultures in the absence of the vitamins at day 7 as described in the text. Then we transferred the cocultures that showed significant growth (more than fourfold increase from the inoculated concentrations) of both the bacterium and the amoeba after the first transfer. The cultivation was stopped at the time points at which the curve truncates. The detection limit of the measurement of amoeba cells was 10^4/mL. These experiments have not been replicated except for the cultures in SIH and SIH′. However, we have checked that the results for the cultures in SIH-(lipoic acid) are robust (see Fig. 4), which is critical for the conclusion. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.2. Measurement of cell concentrations

We measured the cell concentration of the amoeba using a Thoma hemocytometer (Erma Inc., Tokyo, Japan). The cell concentration of the bacterium was determined from the culture's optical density (600 nm) for Fig. 2B, from the number of colony forming units on LB agar plates (Maniatis et al., 1982) for Fig. 3, or from the concentration relative to a known concentration of fluorescent beads (Fluoresbrite YG Microspheres, 3 μm; Polysciences Inc., Warrington, PA, USA) using a fluorescent cell sorter FACSAria (BD Bioscience, CA, USA) for Fig. 4A or a Cytomics FC500 Flow Cytometer (Beckman Coulter, Inc., CA, USA) for Fig. 4C, as described in a previous study (Hosoda et al., 2011).

3. Results and discussion

3.1. The basic design of our SOBEM

The basic design of our SOBEM is shown in Fig. 1. We employed *E. coli* and *D. discoideum* as a model bacterium (Keseler et al., 2011; Neidhardt and Curtiss, 1996) and a model eukaryote (amoeba) (Fey et al., 2007; Kamino et al., 2011), respectively, because experimental ecosystems using this pair of the bacterium and the amoeba had been studied extensively (Kihara et al., 2009, 2011; Matsuyama et al., 2004; Todoriki et al., 2002a,b; Tsuchiya et al., 1972; Yamada et al., 2008). The bacterium used had been genetically engineered to lack the gene *glnA*, which is essential for Gln biosynthesis; thus, the strain does not grow in a medium lacking Gln. For the amoeba, we used a mutant that has only a vegetative growth stage in the life cycle, for simplicity of the system (see Section 2), although wild-type strains of the amoeba also have multicellular development in their life cycle. The amoeba can synthesize Gln but naturally requires a wider variety of nutrients than the bacterium and does not grow if not supplied with one of those essential nutrients. The basic culture medium used for the coculture of the bacterium and the amoeba was a synthetic medium for the amoeba, SIH (Han et al., 2004), which contains all of the nutrients necessary for the growth of the amoeba. Because SIH does not contain Gln, neither the bacterium nor the amoeba grows in monoculture if one of the nutrients, X, which is necessary for the growth of the amoeba, is omitted from SIH (designated as medium SIH-X). However, in coculture, if both organisms complement the lack of the necessary nutrient for the partner, both continuously grow, thereby establishing mutualism. More specifically, the amoeba can supply Gln (or a substitute) by leakage to the medium, and the bacterium can supply the nutrient X (or a substitute) by leakage and/or predation by the amoeba. (These organisms have been used as a model predator-prey system, Tsuchiya et al., 1972.) In this study, we searched for the nutrient (X) that satisfies the following two events: (i) neither the bacterium nor the amoeba grows in monoculture using SIH-X, and (ii) both the bacterium and the amoeba grow in coculture.

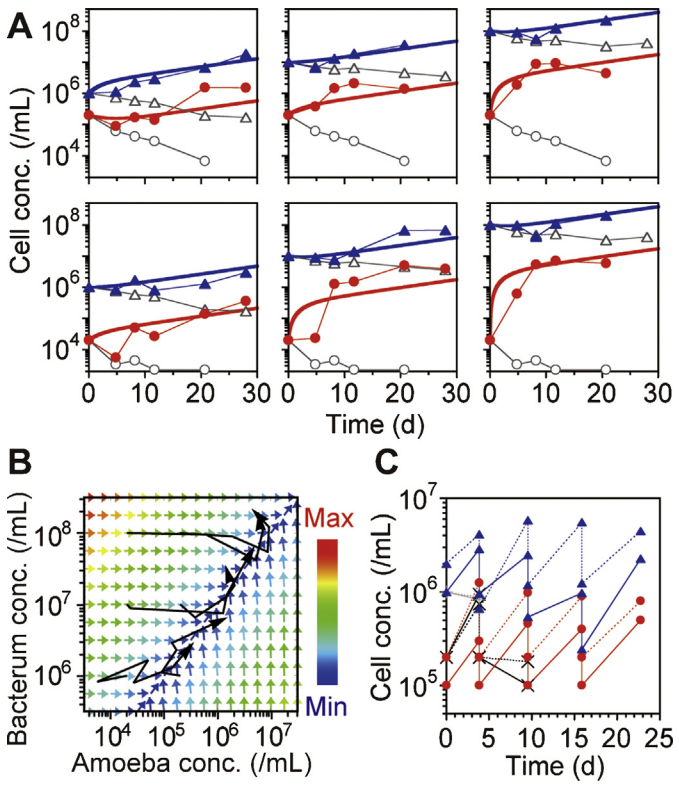

Fig. 4. The population dynamics of coculture in SIH-(lipoic acid). (A) The population dynamics at various initial cell concentrations. The amoeba was precultured once in monoculture with SIH-(lipoic acid) prior to the coculture to avoid the influence of the inertial growth as shown in text. The cell concentrations of the amoeba and bacterium are depicted as red-filled circles and blue-filled triangles, respectively. The population dynamics in the control monocultures of each species are depicted as gray open circles and triangles for the amoeba and the bacterium, respectively. The bold lines indicate the results derived from the model shown in Eq. (1), with a parameter set of $k_A = 2.5$/d, $k_B = 0.0096$/d, $d_A = 0.16$/d, and $d_B = 0.054$/d. The d values were determined from fitting the mortality curves in the monoculture shown as gray points, and the k values were determined from fitting the coculture data to the solution of Eq. (1) using the determined d values. (B) Direction field of the population dynamics. The experimental results of the variation in the cell concentrations shown in (A) (large black arrows) are overlaid with the expected variation at all conditions of the cell concentrations calculated from the mathematical model (small colored arrows). The directions of the colored arrows were calculated as the resultant vector of $(dC_A/dt)/C_A$ and $(dC_B/dt)/C_B$ from Eq. (1), and the colors indicate the relative value of the logarithm of the magnitude of the resultant vector (the minimum and the maximum were set to be purple and red, respectively, as depicted in the figure). (C) Multiple subculturing of the coculture. The red circles and blue triangles indicate the cell concentration of the amoeba and the bacterium, respectively. The solid lines and the dashed lines represent the subculture with different initial amoeba cell concentrations (10^5 and 2×10^5/mL, respectively). The population dynamics in the monocultures of the amoeba and the bacterium (inoculated at 2×10^5 and 10^6/mL, respectively) are depicted as × (black) and + (gray), respectively. The solid lines and the dashed lines show two independent monocultures. The inertial growth of the amoeba in the first culture, and the difference between the monoculture and the coculture after the first transfer are clearly shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Searching for the nutrient X using monoculture

We first searched for the nutrient X that satisfies the following two requirements: the amoeba does not grow in monoculture in SIH-X, and the bacterium grows in monoculture when the SIH-X is supplemented with Gln (SIH-X + Gln). We tested 16 amino acids and 5 vitamins as nutrient X. The results of the amoeba monocultures in SIH-X are shown in Fig. 2A. The amoeba did not show significant growth (more than fourfold increase, *i.e.*, two doublings from the initial concentration) with 11 of the 16 amino acids. For all 5 vitamins, the amoeba showed significant growth in SIH-X when each inoculum was first transferred from SIH (black bars). However, when, thereafter, transferred to the same medium, the amoeba did not show significant growth unless X was cyanocobalamin (white bars). The results of the bacterial monocultures in SIH-X + Gln are shown in Fig. 2B. The bacterium grew unless X was Ile; therefore, Ile could not serve as the nutrient X for which we searched. Although the bacterium did not require Ile in a minimal medium, it is known that Val inhibits the bacterium's growth in the absence of Ile owing to feedback inhibition of an enzyme that catalyzes a reaction in the biosynthesis of both Val and Ile (De Felice et al., 1979). From these results, we selected 10 amino acids and 4 vitamins as candidates for nutrient X: Arg, His, Leu, Gly, Phe, Trp, Lys, Met, Thr, Val, biotin, folic acid, lipoic acid, and riboflavin. All of these nutrients are known to be required for the growth of *D. discoideum* (Franke and Kessin, 1977).

3.3. Searching for the nutrient X by coculturing

We then found that one of these 14 nutrients, lipoic acid can serve as the nutrient X for which we searched. We tested the coculture in media in which each of the selected 14 candidates was omitted. For simplicity, we used SIH′ medium, in which amino acids that are not necessary for the growth of the amoeba (Asn, Asp, Cys, Glu, and Pro) were omitted from SIH (as the basic medium) before the omission of the amino acid candidates. The population dynamics of the cocultures are shown in Fig. 3. The amoeba showed significant growth in both SIH and SIH′ as controls. When one of the 10 amino acid candidates was excluded from SIH′ (*i.e.*, SIH′-X), neither the amoeba nor the bacterium showed significant growth with any of the candidates. In all cases when X was a vitamin, the amoeba showed significant growth upon the first culture after transfer from SIH, as was the case for the monocultures. After transfer to the same medium by subculturing, both the amoeba and the bacterium showed significant growth (more than fourfold increase as above) continuously only when X was lipoic acid. The difference between the monoculture and the coculture is more clearly shown below (Fig. 4). Thus, by examining the 14 nutrient candidates, we found that lipoic acid can serve as the nutrient X for which we searched.

3.4. The stability of the coculture using SIH-(lipoic acid)

The coculture in SIH-(lipoic acid) was continuously cultivatable. First, we analyzed the population dynamics of the coculture in SIH-(lipoic acid) with varying initial concentrations of the amoeba and bacterium (Fig. 4A). The amoeba was precultured once in monoculture with SIH-(lipoic acid) prior to the coculture to avoid the influence of the inertial growth shown above. Under all of the conditions tested, both the amoeba and the bacterium grew continuously in coculture, whereas neither grew in monoculture. More specifically, in coculture, the amoeba first grew to 1/10 the concentration of the bacterium, and then both grew in keeping with their population ratio. We constructed a simple mathematical model to understand the population dynamics of our SOBEM. We assumed that each of the two organisms supplied the nutrient (or a substitute) at a constant rate per cell and that the entire amount of the supplied nutrient was transferred to the cell body of the partner. Based on those assumptions, we formulated the equation for the population dynamics as follows:

$$\frac{dC_A}{dt} = k_B C_B - d_A C_A, \qquad \frac{dC_B}{dt} = k_A C_A - d_B C_B, \tag{1}$$

where the subscripts A and B denote the amoeba and the bacterium, respectively, C_Y, k_Y, and d_Y are the cell concentration of Y, the rate constant of the nutrient supply from a single cell of Y,

and the mortality rate of the *Y* cells, respectively. We used only simple linear terms in the model and did not include specifically assigned non-linear terms such as that for predation because we did not identify the main cause for the interactions. The time-varying functions C_A and C_B in the model can be solved analytically, and we found that this extremely simple model provided a reasonable description of the experimental data for the population dynamics by using a common set of the constants (Fig. 4A, bold lines). Moreover, the direction field (Hass et al., 2007) in Fig. 4B, which gives a visualization of the mathematically predicted general shape of the population dynamics overlaid by the experimental results, clearly shows that the stable population ratio is reasonable, suggesting the ratio C_B/C_A will become approximately 10 even when we inoculate the cells at another initial cell concentrations. Note that we do not argue that the interactions between the two organisms were only due to exchanges of leaked glutamine and lipoic acid via the culture media. The interaction could be due to exchanges of other compatible substances, predation, or pleiotropic effects. We then cocultured the organisms with the initial concentrations providing a 10:1 population ratio of bacterium to amoeba, and then, before either population reached saturation, we transferred the coculture into the fresh medium, making the initial concentration of the amoeba become 10^5 or 2×10^5/mL (Fig. 4C). The coculture was continuously cultivatable; namely, the SOBEM was established with a stable population ratio.

4. Conclusion

We constructed synthetic obligate mutualism between a model bacterium and a model eukaryote via coculture. The constructed mutualism was induced by simple syntrophy of essential nutrients between two organisms that would rarely interact in nature. This first construction of synthetic mutualism between a bacterium and a eukaryote sheds light on the establishment of bacteria–eukaryote mutualism in nature. It is also remarkable that the two species are normally capable of a predator–prey interaction, but they can also readily form mutualisms. Moreover, the organisms established the novel mutualism under one of the 14 conditions tested, comparable to previous reports of synthetic mutualism between organisms other than the pair of bacteria and eukaryotes (Hillesland and Stahl, 2010; Hosoda et al., 2011; Hosoda and Yomo, 2011; Shendure et al., 2005; Shou et al., 2007; Wintermute and Silver, 2010b). Thus, our results add to the body of knowledge on mutualism and may suggest that an organism in nature has a low but non-negligible possibility of establishing novel mutualism by merely encountering another potentially mutualistic species, regardless of the pair of their kingdoms to which they belong. Although we show that our SOBEM was continuously cultivatable and that the population dynamics were stable, we do not argue that our SOBEM is evolutionarily stable or sustainable against the evolutionary vulnerability of mutualism. Because the way in which mutualism evolves is one of the principal questions in ecology and evolution, further study on the experimental evolution of this SOBEM is important. Moreover, because our SOBEM involved phagocytic processes, the experimental evolution of this SOBEM would be expected to move toward synthetic endosymbiosis, which is an ambitious, long-term endeavor (Agapakis et al., 2011; Jeon, 1972; Maurino and Weber, 2013; Nakajima et al., 2009).

Acknowledgments

We thank Drs. Mineko Maeda and Hidekazu Kuwayama for providing *D. discoideum* strains. We thank Drs. Satoshi Sawai and Akiko Kashiwagi for helpful discussions. We are also grateful to Ms. Naomi Tago, Ms. Ryoko Otsuki, and Mr. Kenji Tomita for their valuable technical assistance. This study was partially conducted in the Open Laboratories for Advanced Bioscience and Biotechnology (OLABB), Osaka University, Japan. This research was supported in part by the "Global COE Program" and the "Special Coordination Funds for Promoting Science and Technology: Yuragi Project" of the Japanese Ministry of Education, Culture, Sports, Science and Technology, JSPS KAKENHI Grant Number 23770021, and "Novel and innovative R&D making use of brain structures" from the Ministry of Internal Affairs and Communications Japan.

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